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Adenine nucleoside diphosphates block adaptation of mechanoelectrical transduction in hair cells

(auditory system/ear/molecular motor/myosin/vestibular system)

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ABSTRACT **By adapting to sustained stimuli, hair cells in the internal ear retain their sensitivity to minute transient displacements. Because one model for adaptation asserts that this process is mediated by a myosin isozyme, we reasoned that we should be able to arrest adaptation by interfering with myosin's ATPase cycle though introduction of ADP into hair cells. During tight-seal, whole-cell recordings of transduction currents in cells isolated from bullfrog (*Rana catesbeiana*) sacculus, dialysis with 5–25 mM ADP gave variable results. In half of the cells examined, the rate of adaptation remained unchanged or even increased; adaptation was blocked in the remaining cells. Because we suspected that the variable effect of ADP resulted from the conversion of ADP to ATP by adenylate kinase, we employed the ADP analog adenosine 5′-[β-thio]diphosphate (ADP[βS]), which is not a substrate for adenylate kinase. Adaptation consistently disappeared in the presence of 1–10 mM ADP[βS]; in addition, the transduction channels' open probability at rest grew from ≈0.1 to 0.8 or more. Both effects could be reversed by 2 mM ATP. When used in conjunction with the adenylate kinase inhibitor P^1,P^5-bis(5′-adenosyl) pentaphosphate (Ap_5A), ADP had effects similar to those of ADP[βS]. These results suggest that adaptation by hair cells involves adenine nucleotides, and they lend support to the hypothesis that the adaptation process is powered by a myosin motor.**

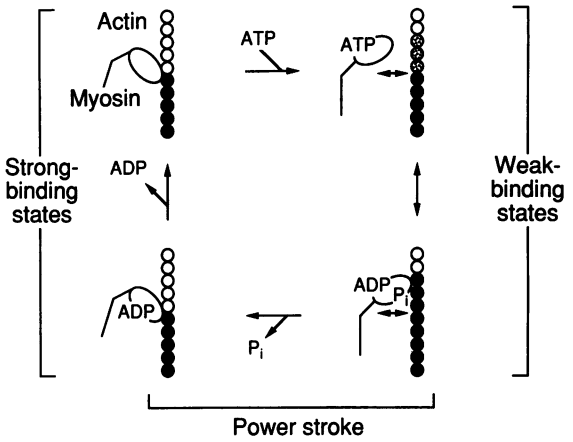

FIG. 1. Schematic depiction of myosin's ATPase cycle. In the nucleotide-free or rigor state, the myosin molecule is tightly bound to an actin filament. ATP binds to myosin and promotes its rapid dissociation from the filament; during the ensuing weak interaction with actin, myosin cleaves ATP to ADP·P_i. As P_i is released, the myosin molecule rebinds tightly to the actin filament and performs a power stroke. Finally, ADP dissociates and the rigor state recurs. Because myosin and actin interact with high affinity, the rigor and ADP-bound states are both capable of sustaining mechanical force.

By responding to sounds and accelerations, the internal ear performs one type of mechanoelectrical transduction. Hair cells, the receptors of the cochlea and vestibular labyrinth, transform mechanical stimuli into electrical signals (reviewed in refs. 1 and 2). The electrical response of each such cell is produced by deflection of a mechanically sensitive organelle, the hair bundle, which emerges from the hair cell's apical surface. A hair bundle comprises 20–300 actin-stiffened stereocilia, clustered together like the staggered pipes of an organ, and a single true cilium, the kinocilium. When a hair bundle is displaced, transduction channels, perhaps as few as one per stereocilium (3–5), are directly pulled open by tension in gating springs within the hair bundle (6). Because these channels occur at the bundle's top (7, 8), it is probable that each gating spring is the thin, filamentous tip link that extends from the end of a stereocilium to the side of its tallest neighbor (9, 10).

When a hair bundle is subjected to a protracted displacement, the cell's response adapts (11–16): the bundle's range of responsiveness migrates toward the position to which the bundle has been moved. Adaptation is associated with mechanical changes within the hair bundle that are thought to reflect adjustment of the tension in gating springs (12, 17). One model for adaptation posits that the tension in each gating spring is maintained by motor molecules whose activity is regulated by the cytoplasmic Ca^{2+} concentration (12, 16, 18; reviewed in ref. 2). During adaptation to positive stimuli, it is supposed that Ca^{2+} enters a stereocilium through open transduction channels and causes the motor to slip down a microfilamentous track, relieving tension in the gating spring. During negative stimuli, when transduction channels are closed and the cytoplasmic Ca^{2+} concentration falls, the motor climbs up the track to restore tension. Because it could move along the microfilaments that form the cytoskeletal core of a stereocilium (19), a member of the myosin family might well serve as the adaptation motor (12, 18, 20).

To test the hypothesis that myosin molecules are responsible for adaptation in the hair cell's transduction process, we sought to arrest the motors upon their tracks. A hair cell with its adaptation motors stalled would be incapable of adjusting tip-link tension and should therefore maintain a constant transduction current in response to a protracted bundle displacement. Tailoring our approach to the ATPase cycle of skeletal-muscle myosin (Fig. 1; reviewed in refs. 21 and 22), we sought to arrest a myosin molecule in either of two states in the cycle: rigor, in which the myosin molecule has no bound nucleotide, or the ADP-bound state, which ensues after release of inorganic phosphate ion (P_i). A myosin molecule confined to either of these states would be tightly bound to the actin filament, able neither to climb nor to slip.

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Abbreviations: ADP[βS], adenosine 5′-[β-thio]diphosphate; Ap_5A, P^1,P^5-bis(5′-adenosyl) pentaphosphate.

Our results, some of which have appeared in preliminary form (23), demonstrate that ADP or an ADP analog blocks adaptation, and thus implicate adenine nucleotides in adaptation. Furthermore, the characteristics of the inhibition of adaptation are entirely consistent with the suggestion that the process is myosin based.

MATERIALS AND METHODS

Adenine Nucleotide Solutions. Adenine nucleotides were obtained from Boehringer Mannheim. The concentrations of ATP, ADP, and adenosine 5′-[β-thio]diphosphate (ADP[βS]) were determined by their absorbances at 259 nm on the basis of a molar extinction coefficient of $1.54 \times 10^6\ M^{-1}\cdot m^{-1}$ (24). We estimated the concentration of P^1,P^5-bis(5′-adenosyl) pentaphosphate (Ap_5A) by assuming twice that molar extinction coefficient. The purity of the nucleotides was established by high-performance liquid chromatography on an anion-exchange column (Mono Q, Pharmacia/LKB) with a 15-ml gradient of 0–1 M ammonium bicarbonate. Although the purities of ATP and ADP exceeded 95%, ADP[βS] was contaminated by AMP (15%) and a small amount of ADP (0.1%).

Electrophysiological Recording. Hair cells were isolated from bullfrog (*Rana catesbeiana*) sacculus after exposure of the organ *in situ* to low-Ca^{2+} saline solution (10, 16). In a modification of the published procedure, 1 mM EGTA and 1 mM $MgCl_2$ replaced 1 mM EDTA in the solution dripped into the perilymphatic cistern. In addition, DNase treatment was omitted and cells were mechanically dissociated in saline solution containing 100 μM $CaCl_2$.

After solitary hair cells were firmly adsorbed onto a concanavalin A-coated coverslip in an experimental chamber, they were maintained at 21°C in an oxygenated saline solution containing 110 mM Na^+, 2 mM K^+, 4 mM Ca^{2+}, 118 mM Cl^-, 3 mM D-glucose, and 5 mM Hepes at pH 7.25. Cells were observed through a 63× objective lens and Nomarski differential-interference-contrast optics on an inverted microscope (Zeiss model IM-35) with a rotating stage.

Transduction currents were recorded with whole-cell tight-seal pipettes (25), whose axial resistances were 3–4 MΩ prior to seal formation and generally rose to 10–20 MΩ during recording. All internal pipette solutions contained 85 mM CsCl, 1 mM $MgCl_2$, 1 mM EGTA, and 5 mM Hepes. After addition of adenine nucleotide with an equimolar concentration of $MgCl_2$, an internal solution's pH was adjusted to 7.25–7.30 with CsOH. Recordings were made at a holding potential of −74 mV, which took into account dissipation of a +4-mV tip potential between a pipette's contents and the bath solution. Because ADP[βS] was supplied as its lithium salt, we performed several control experiments with 10 mM LiCl or 20 mM LiCl plus 2 mM ATP added to the internal solution; transduction and adaptation in these instances were similar to those under ordinary conditions.

In some experiments, we filled the tip of the recording electrode with a solution different from that in the pipette's shank. To ensure that the shank solution was capable of diffusing into a cell in only a few minutes, we dipped the electrode's tip into the appropriate solution for only 1–2 s. Control experiments with dye-containing solutions indicated that this procedure provided ≈2 nl of fluid at the pipette's tip.

Stimuli were delivered with a micropipette, which was attached to the kinocilium's bulbous tip by gentle suction (3) and displaced by a piezoelectrical stack (PZL-007-1, Burleigh Instruments, Fishers, NY). We monitored the time course and extent of adaptation to 100-ms bundle displacements of up to ±500 nm. For the construction of displacement-response relations, cells were subjected to a range of 16-ms deflections of amplitudes up to ±1050 nm. The peak transduction currents were fitted with a Boltzmann relation (4, 6),

$$p = \frac{I}{I_{max}} = \frac{1}{1 + e^{-z(X-X_0)/kT}}.$$

Here p is the channels' open probability, I is the transduction current in response to a hair-bundle displacement X, I_{max} is the maximal transduction current, z is the channels' sensitivity to displacement, and X_0 is the displacement at which half the channels are open; k is the Boltzmann constant and T is the thermodynamic temperature. We used the fitted data to estimate the fraction of channels open with the hair bundle in its resting position. To demonstrate that changes in the channels' open probability were not artifactually produced by drift of the stimulus probe, recording electrode, or microscope stage, we occasionally detached the probe and reattached it after confirming that the bundle was in its resting position.

Transduction currents were measured with an amplifier (EPC-7, List Electronics, Darmstadt, Germany) operated without series-resistance compensation. Stimulation was controlled and responses were recorded by means of an experimental interface (Indec Systems, Sunnyvale, CA) whose operation was programmed in BASIC-23 on a computer (PDP-11/73, Digital Equipment). The experimental environment has been described in detail (8).

RESULTS

We used whole-cell, tight-seal electrodes to record transduction currents from hair cells isolated from the bullfrog's sacculus. When a pipette contained no nucleotide or millimolar concentrations of ATP, our results accorded with those from previous investigations of hair cells from this and other organs (reviewed in ref. 1). With the hair bundle in its resting position, the cell's mechanically sensitive channels bore an inward transduction current that was ≈15% of the maximum that could be evoked; the channels' open probability was thus ≈0.15 (3). Moving the hair bundle progressively in the positive direction, towards its tall edge, opened additional channels until it evoked a transduction current that could be as great as −375 pA. Negative stimuli, on the other hand, closed some or all of the channels that were open at rest (Fig. 2*A*).

During a given displacement, the transduction current did not persist at a constant amplitude (11, 13, 14, 16). Instead, as adaptation occurred in response to a positive displacement, the response declined towards the resting level. Adaptation also proceeded during a negative displacement; the amplitude of the current transient following such a stimulus revealed the degree of adaptation.

When the recording pipette contained millimolar concentrations of ATP, adaptation was robust (Fig. 2*A*) and endured for as long as the cell was healthy, generally for more than 15 min. When the pipette contained no nucleotide, a cell's ability to adapt was labile, but nevertheless often persisted for several minutes. If ATP is required for the activity of an adaptation motor, the cells from which we recorded evidently contained a substantial reservoir of the compound or retained the capacity to produce more.

Because we anticipated difficulty in extensively lowering the cellular ATP concentration through a micropipette (26), we attempted instead to arrest the putative hair-bundle motors by dialyzing hair cells with ADP. In half (6 of 12) of the hair cells dialyzed with 5–25 mM ADP, adaptation was, as expected, blocked within a few minutes (data not shown). In the remaining 6 cells, by contrast, adaptation persisted or even accelerated; several cells retained robust transduction and adaptation for longer than 20 min. Because such enduring adaptation was reminiscent of the finding in control cells dialyzed with ATP, we suspected that adenylate kinase, the

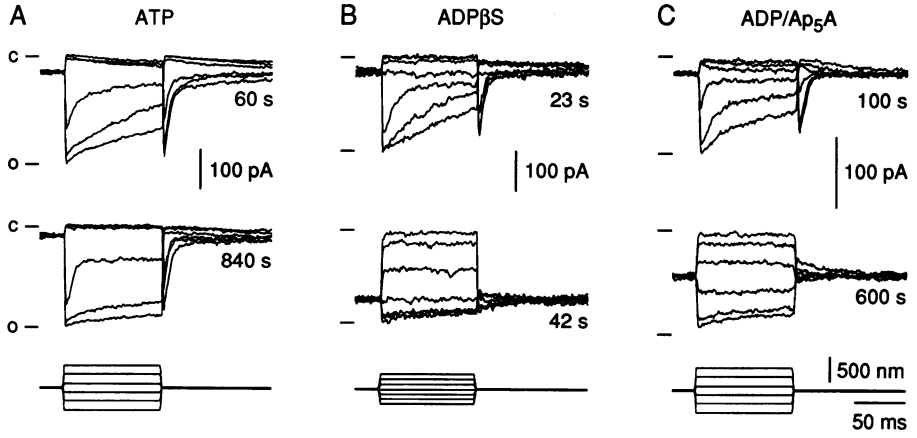

FIG. 2. Arrest of adaptation by ADP[βS] or by ADP and Ap_5A. Families of transduction-current records were elicited by 100-ms hair-bundle displacements of the magnitudes shown in the bottom traces. In this and subsequent figures, the time indicated beside a record represents the interval from the onset of whole-cell recording until collection of the displayed data commenced. For each set of traces, a pair of fiducial marks indicates the current with all the channels closed (labeled c in part *A*) or open (labeled o). (*A*) During recording with a pipette filled with solution containing 2 mM ATP, brisk adaptation persisted for more than 14 min. Ten traces were averaged for the set of responses obtained at each time. (*B*) When the recording pipette contained 10 mM ADP[βS], adaptation vanished as the nucleotide diffused into a cell. During the same period, the probability that the transduction channels were open at rest increased from 0.17 to 0.80. Because of the rapidity of the ADP[βS] effect, only three traces were averaged at each time. (*C*) A similar effect was observed when the recording pipette contained 5 mM ADP and 250 μM Ap_5A. In this instance, the recording was begun with 2 mM ATP in the pipette's tip, and adaptation was gradually arrested as that nucleotide was replaced by diffusion of ADP and Ap_5A from the pipette's shank. Each record represents the average of 10 traces.

ubiquitous enzyme that catalyzes the interconversion of adenine nucleotides (27), continuously produced sufficient ATP in ADP-filled hair cells to support adaptation.

To preclude adenylate kinase activity, we dialyzed hair cells with ADP[βS], an ADP analog that is not a substrate for the enzyme (28). In 26 of 29 cells filled with 10 mM ADP[βS], adaptation was dramatically reduced during both positive and negative bundle displacements (Fig. 2*B*). The loss of adaptation usually occurred promptly; in most cells, adaptation vanished within 60 s of establishment of the whole-cell recording configuration (Fig. 3*A*). Adaptation was also arrested within 120 s in four of five cells dialyzed with 1 mM ADP[βS]. For each of the four cells that did not lose adaptation when filled with 1–10 mM ADP[βS], the recording pipette's access resistance was unusually high. When six cells were filled with 100 μM analog, two cells showed no effect, and blockage required 200–500 s in the remainder. At a concentration of 1 mM or greater, ADP[βS] thus appeared to stall the adaptation motors in the manner expected.

As it arrested adaptation, ADP[βS] treatment consistently caused an additional change in a hair cell's mechanosensitivity: the transduction channels' probability of being open with the bundle in its resting position increased from ≈0.1 to 0.8 or more. The displacement-response relation, which characterizes the probability that channels are opened by stimuli of various amplitudes, consequently shifted in the negative direction (Fig. 4). After the channels' open probability at rest exceeded 0.8, it sometimes fell spontaneously to 0.5 or less. The latter phenomenon occurred more frequently in cells dialyzed with 0.1–1 mM ADP[βS] than in those exposed to a higher concentration of the nucleotide.

In many cells dialyzed with ADP[βS], transduction suddenly disappeared several minutes after the arrest of adaptation. In several other instances, the conventional response abruptly vanished, but a transient current, presumably borne by transduction channels, could still be elicited (Fig. 3 *B* and *C*).

We demonstrated in two cells that the effects of ADP[βS] could be almost entirely reversed by ATP. When a pipette's tip was filled with 10 mM ADP[βS] and its shank with 2 mM ATP, adaptation was blocked shortly after the onset of recording. As ATP subsequently diffused into the cell, however, adaptation resumed and the resting open probability returned to approximately its control value (Fig. 5).

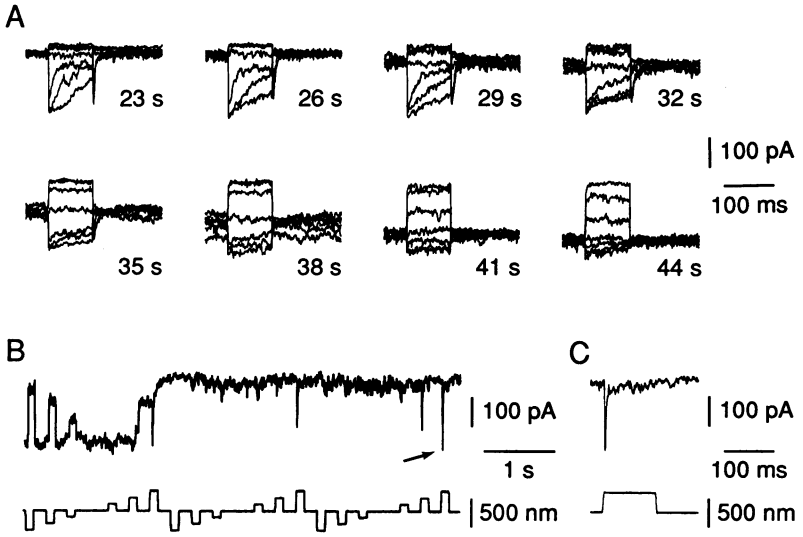

FIG. 3. Time course of the effects of ADP[βS] on transduction and adaptation by a single cell. (*A*) At each of the indicated times, families of transduction currents were measured with the stimulus paradigm displayed in Fig. 2*B*. The reduction in the rate of adaptation and the increase in the channels' resting open probability developed continuously and rapidly. Individual records are displayed. (*B*) After acquisition of the last record shown in *A*, transduction and adaptation were abruptly and almost entirely lost; thereafter, transient inward currents were sporadically elicited by positive stimuli. (*C*) Displayed on a faster time base, a portion of the record shown in *B* (indicated by an arrow) revealed the transient nature of the residual current.

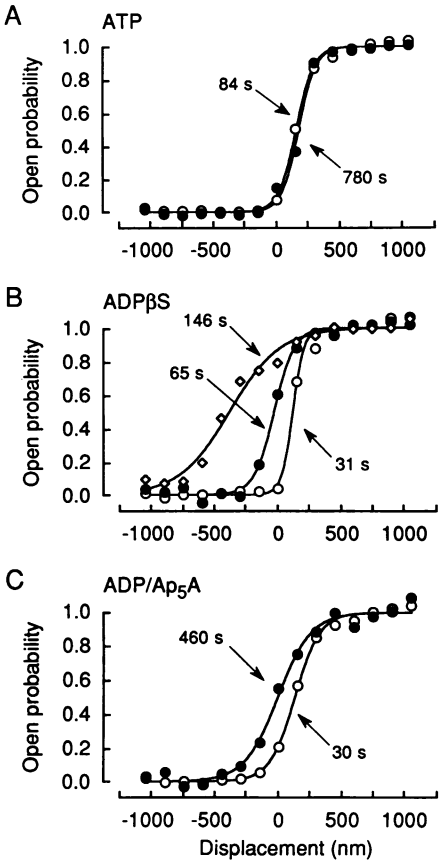

FIG. 4. Displacement–response relations obtained during dialysis of hair cells with 2 mM ATP (*A*), 10 mM ADP[βS] (*B*), or 5 mM ADP and 250 μM Ap_5A (with the pipette's tip initially filled with 2 mM ATP) (*C*). The displacement–response relation shifted in the negative direction and became shallower in the presence of ADP[βS]; both effects were much less pronounced with the mixture of ADP and Ap_5A. Fits of the data shown in *B* with a Boltzmann relation indicated that X_0, the point at which half of the channels were open, shifted from +120 nm to −381 nm as ADP[βS] filled the cell. In addition, z, the single-channel gating force and a measure of the curve's slope, declined from 89 to 20 fN. This decreased slope may have reflected dispersion of the sensitive ranges of individual transduction elements (1). The maximal transduction current was −102 pA at the recording's outset and −98 pA at its conclusion.

Another potential means of avoiding the effects of adenylate kinase is to inhibit the enzyme. Some isozymes of adenylate kinase are potently inhibited by Ap_5A (29). In each of the seven cells that we successfully dialyzed with a combination of 5 mM ADP and 250 μM Ap_5A, adaptation slowed over 120–600 s (Fig. 2*C*). Although with this treatment the open probability at rest again rose, its value rarely exceeded 0.5 (Fig. 4*C*). An additional, unexplained, effect was observed: adaptation was usually absent immediately after the whole-cell recording configuration was established with a pipette containing ADP and Ap_5A but then recovered over a minute or so. This transient effect was evidently due to extracellular leakage of Ap_5A as the pipette approached the cell. To avoid this complication, we made recordings in which a pipette with similar contents was filled at its tip with a few nanoliters of 2 mM ATP. In each of four experiments, the initial interruption of adaptation was then absent, and the subsequent decline of adaptation resembled that observed with ADP[βS] in the pipette solution (Fig. 2*C*).

DISCUSSION

The interruption of adaptation by adenine nucleoside diphosphates displayed characteristics consistent with the hypothesis that adaptation is effected by a myosin isozyme. Adaptation was rapidly and completely blocked as ADP[βS] diffused into a cell; in addition, ADP often had a similar effect. Because mechanoelectrical transduction itself remained robust, it appeared as if the adaptation mechanism had been selectively arrested. The transduction channels' probability of being open at rest increased, which likely reflected greater tension applied to the gating springs by adaptation motors. This phenomenon is consistent with a feature of conventional myosin isozymes: in isometrically contracting skeletal muscle provided with ATP, tension increases by as much as 50% upon exposure to millimolar concentrations of ADP (30, 31). Because myosin molecules sustain force in tightly bound states, this effect is thought to reflect an augmented population of myosin molecules trapped in force-producing states after their power strokes (31). Because the transduction channels' probability of being open at rest depends on the tension in gating springs (10, 17), an increase in the population of the ADP-bound state might well raise the open probability. In the cell whose mechanical sensitivity was characterized by the Boltzmann relation presented in Fig. 4*B*, it may be calculated (32) that a 50% increase in gating-spring tension would have raised the channels' open probability at rest from $\approx$0.1 to >0.9.

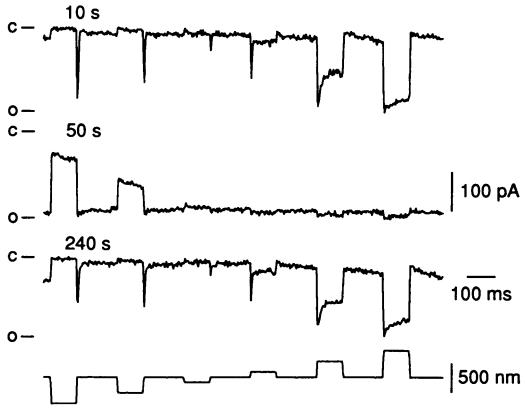

FIG. 5. Reversal by ATP of the effects of ADP[βS] on transduction and adaptation. Transduction currents were recorded with a pipette whose tip was filled with 10 mM ADP[βS] and shank with 2 mM ATP. Ten seconds after establishment of the whole-cell configuration, adaptation was normal. After 50 s of recording, adaptation was mostly blocked and the channels' resting open probability was 0.92. Later in the recording session (240 s), the effects of ADP[βS] were largely reversed as ATP diffused into the cell from the pipette's shank. Three sets of transduction currents were averaged in each case. During the final record displayed, and still more strikingly at times between the 50-s and 240-s records, the channels' open probability systematically increased after successive positive stimuli. The open probability then reverted to a lower value after a large negative stimulus.

If the increase in open probability resulted from enhanced tension in gating springs, it should have been accompanied by movement of an unconstrained hair bundle in the negative direction (16). The expected motion (32), however, would have been only some −70 nm. Because of the drift inherent in present displacement-measuring systems (12, 16, 17), a movement of this magnitude over a period of many seconds cannot yet be detected.

After protracted dialysis of a cell with ADP[βS], ordinary mechanical responses sometimes vanished and the resting open probability approached zero (Fig. 3*B*). This phenomenon resembles that observed upon exposure of hair bundles to an extracellular Ca^{2+} concentration below $\approx$20 μM (5), which results in the destruction of tip links (10), the presumptive gating springs. Because ADP[βS] treatment sometimes spared intermittent and transient responsiveness to

mechanical stimulation (Fig. 3C), however, the nucleotide was unlikely to have destroyed the tip links. What, then, might have been the source of the residual responses? It is plausible that the motor associated with each tip link contains several myosin molecules (17, 32). After ADP[βS] had inactivated many of these, enhanced tension in the tip links may well have torn the motors loose from their cytoskeletal attachments. Transient currents might then have arisen whenever evanescent rebinding of a few myosin molecules allowed the gating springs to sustain some tension.

The combination of ADP and Ap_5A inhibited adaptation considerably more slowly than did ADP[βS]. It is possible that the concentration of Ap_5A employed, although substantial, was nonetheless insufficient to fully inhibit the hair cell's adenylate kinase. Soluble adenylate kinases are blocked by Ap_5A with inhibition constants (K_i values) of 2–20 nM with respect to ADP (29, 33, 34), but mitochondrial isozymes are much less sensitive to the compound, with inhibition constants of 2–6 μM (34, 35). If Ap_5A is a competitive inhibitor with respect to ADP (35), the 20-fold higher concentration of ADP in our internal solution may have promoted the synthesis of enough ATP to support adaptation.

In the light of the arguments presented above, it seems peculiar that hair cells can retain adaptation for minutes when dialyzed with internal solutions lacking adenine nucleotides altogether (13, 14). If the initial cytoplasmic concentration of ATP was ≈1 mM (36), however, dialysis of a hair cell through pipettes such as those we employed should have left the ATP concentration above ≈100 μM for at least 10 min (26). This concentration is sufficient to sustain many ATP-dependent processes, including myosin-based motility (30).

It is appropriate to inquire whether other possible mechanisms of adaptation, besides that invoking myosin, could explain our results. Because ADP[βS] may inhibit adenylate kinase (28), any cellular process dependent upon the enzyme's activity might have been blocked. A reduced intracellular Ca^{2+} concentration, such as that produced by depolarization or by lowering the extracellular Ca^{2+} concentration, also diminishes the rate of adaptation and shifts the displacement–response relationship (13, 14, 16). Because the amplitude of the transduction current was usually unaffected by ADP[βS] or by ADP and Ap_5A, however, it is unlikely that the compounds blocked the only known route of Ca^{2+} entry into the hair bundle, that through the transduction channels (37, 38). It is also improbable that ADP[βS], ADP, or Ap_5A accelerated Ca^{2+} extrusion by a pump or exchanger. In particular, the ATP requirement of Ca^{2+}-ATPase implies that the nucleotides would more likely have inhibited Ca^{2+} removal by a pump. The effects of the nucleoside diphosphates are also difficult to reconcile with an alternative model of adaptation that involves stabilization of a closed state of the channel by Ca^{2+} (14). Finally, microtubule-based motor proteins such as kinesin and dynein cannot be responsible for adaptation; the only microtubules found in the hair bundle occur in the kinocilium, which is unnecessary both for transduction (39) and for adaptation (16). Although adaptation might be effected by an unknown process that exhibits the characteristics reported here, the hypothesis that a myosin isozyme mediates adaptation most parsimoniously fits our data.

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